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A MICROMETHOD FOR DETERMINING POLYUNSATURATED FATTY ACIDS; ITS CLINICAL AND EXPERIMENTAL APPLICATIONS

by

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1. INTRODUCTION

Among various fatty acids, those having two or more double bonds in the carbon chains are called polyunsaturated fatty acids (abbreviated PUFA). According to the number of double bonds, PUFA are classified into dienoic acid (Di), trienoic acid (Tr), tetraenoic acid (Tt), pentaenoic acid (Pt) and hexaenoic acid (Hx). These polyenoic acids each have many isomers. It is believed that natural Di is composed mostly of linoleic acid, Tr is composed mostly of linolenic acid and Tt is composed mostly of arachidonic acid. Natural Pt is composed of eicosapentaenoic acid and docosapentaenoic acid¹⁾. Natural Hx is composed mostly of docosahexaenoic acid²⁾.

These PUFA have a very important nutritional significance distinct from that of the more abundant saturated and monounsaturated acids, which are associated with them in nature. Although PUFA are indispensable for maintaining physiological functions, they are not entirely synthesized in animal bodies. Therefore PUFA should be included in the diet^(3)~10). Each PUFA undergoes some changes in animal bodies, and if either linoleic acid or arachidonic acid and either linolenic acid or docosahexaenoic acid are satisfactorily supplied, the animal does not develop the PUFA-deficiency syndrome¹⁰⁾. Pt seems to be a metabolic product of an other PUFA, for example linolenic acid^{10)~12)}. Among these PUFAs, arachidonic acid is recognized as the most important substance for biochemical processes^{13)~15)}.

In our Division, using a fat emulsion which can be safely administered intravenously for the purpose of parenteral nutrition, it was discovered that fat is not only a source of calories but also has a specific nutritional effect of promoting wound healing and of protecting against postoperative complications^{16)~19)}. This specific effect might be the action of the PUFAs in the emulsion of sesame oil which was used for clinical and experimental purposes. This action was investigated by paper chromatography of fatty acids, and it was discovered that the effect of the PUFA in the sesame oil emulsion must not be neglected¹⁸⁾²⁰⁾. However, the microanalysis of PUFA still has many defects, and a high reproducibility has not yet

been obtained.

There are many ways to analyze PUFA quantitatively. Salt formation, bromination, fractional distillation and low temperature crystallization have been used, but these methods required large samples for analysis and specialized equipment were too complicated for clinical use²¹⁾.

Several methods of quantitative analysis of PUFA in small samples, applicable to clinical measurement have been developed. Those now in use are chromatography²⁰⁾²²⁾ and spectrophotometry^{23)~31)}. But these two methods have merits and demerits. The former is adequate to discriminate between acids with the same chain length or same number of double bonds, but not adequate to differentiate between the acids of a mixture. On the contrary, the latter have the merit of being able to differentiate between PUFAs in a complicated mixture as to the number of double bonds, but it can not differentiate between acids with the same number of double bonds and different chain lengths. But because the kinds of natural PUFAs are limited as mentioned above, the errors of spectrophotometry of natural PUFA were relatively small and the method is at present the best for the clinical measurement of PUFA.

Spectrophotometry of PUFA has been improving since MOORE²³⁾ developed the method, and highly unsaturated acids can also be measured now. The accuracy was also improved, and microanalysis was conceived^{24)~31)}. But the accuracy of these methods is not yet good enough for clinical application, and several modifications were needed. This was discovered when the author determined PUFA of various natural lipids by spectrophotometry using especially the method of HOLMAN and HAYES²¹⁾³⁰⁾.

Therefore, the method was modified by the author. The determination of PUFA in various tissues of tumor-bearing patients and animals proved that this method could be used in the clinical determination of PUFA and that it is an excellent method with high accuracy.

As PUFA are indispensable foodstuffs, the consumption of PUFA may be increased in tumour tissues which have very rapid cell multiplication. HAVEN discovered in 1937 that the iodine value of lipids in muscles of rats with implanted tumours was lower than that in normal rats, and he believed that this was due to decreased PUFA³²⁾. In 1939 MIURA and SUEYOSHI discovered that dienoic acid was decreased in the tissue of myosarcoma and dienoic acid and trienoic acid were decreased in the tissue of myoma. There was no change in tetraenoic acid³³⁾. In 1941 SMEDLEY-MACLEAN and NUNN investigated fatty acids in rats with implanted WALKER tumour, and obtained results which were not very definite³⁴⁾³⁵⁾. These facts indicate that analytical methods used to date have not been very effective. With the author's modification of the method of HOLMAN and HAYES, constant results were obtained under certain definite conditions.

The author's method is as follows, and the improved points are discussed. Then the changes of PUFA are described in tissues of patients and rats under various conditions.

II. METHOD OF DETERMINATION

One of the methods now widely used for determining PUFA is spectrophotometric analysis after alkaline isomerization. And, among many modifications of the method, that of HOLMAN and HAYES is the best for clinical measurement as stated above^{(21)~(30)}. The following experiments were carried out in accordance with their method.

a. Apparatus

A spectrophotometer equipped with quartz or fused silica cells having a light path of 1.0 cm, a microbalance, a constant temperature apparatus maintaining $180^{\circ} \pm 0.5^{\circ}\text{C}$ (may consist of a hot oil bath), a distillation apparatus at reduced pressure and equipment for supplying nitrogen are needed for the determination. The reaction vessels are 16×150 mm telex-glass test tubes marked at 5.0 ml. The vessels are provided with nitrogen inlet tubes which cover the tops and introduce nitrogen gas over the reagent and sample. To make the ethylene glycol reagent, a 200 ml flask made of telex-glass was used, equipped a nitrogen inlet tube and a thermometer of 200°C .

b. Reagents

Potassium hydroxide in ethylene glycol was used to isomerize the fatty acids. One hundred and two grams of ethylene glycol (reagent grade) was measured in a 200 ml flask made of telex-glass (2% may be evaporated during the process), and 28.0 g of potassium hydroxide (85% analytical reagent grade) was added. The mixture was heated in an oil bath to 190°C under nitrogen and maintained at this temperature for several minutes, then cooled in cold water to room temperature. The reagent must be stored in glass stoppered bottles under nitrogen below 5°C . Thus stored, the reagent can be used for one month.

Before use, the concentration must be examined titrimetrically, and if it is not $21.0 \pm 0.1\%$ it should be made again freshly. Usually, it was easy to gain the concentration by keeping a constant flow of nitrogen gas. If the flow is too rapid, the evaporation of solvent increases and the terminal concentration may be too high. And if the flow is too slow, the reagent may be coloured. A nitrogen flow of 2~3 ml per second was used in the experiment.

The solvents required for the extraction of lipids and in the analysis were peroxide free ethyl ether, 95% ethanol (redistilled), light petroleum ether (B. P. $20^{\circ} \sim 40^{\circ}\text{C}$) and methanol (reagent grade). The methanol should have a density of less than 0.3 at 220 $m\mu$ when measured against water. 3:1 ethanol-ether was prepared 3 parts of ethanol described above being added to one part of ethyl ether just before use. Acidic ethanol-ether was prepared by adding 5 parts of concentrated hydrochloric acid to 95 parts of freshly prepared ethanol-ether, 3 to 1.

c. Extraction

Usually 2.00 ml of serum or plasma was added with rapid mixing to 10 ml of ethanol-ether, which then stood overnight at room temperature. For the extraction of tissue, about 0.1 g of liver, heart, kidney etc. or about 0.05 g of adrenal

should be excised. The excised samples were weighed exactly by microbalance and macerated in 10 ml of acidic ethanol-ether, then they stood overnight. The mixture of serum or tissue was filtered through a BÜCHNER funnel with suction, and the precipitate was washed twice with 5 ml portions of ethanol-ether and once with 10 ml of light petroleum ether on the funnel. The combined extracts were transferred to a separatory funnel. Ten ml of 0.5% saline solution was added and the content were shaken, and the petroleum ether layer was separated. The aqueous layer was extracted twice more with 5 ml portions of light petroleum ether. The petroleum ether extracts were combined and washed twice with 10 ml portions of distilled water and dried with anhydrous sodium sulfate. The precipitate was washed after filtration twice with 5 ml portions of light petroleum ether. The dried extract was evaporated down to about 5 ml in a pear-shaped flask on a water bath at 60°C under nitrogen at reduced pressure. The concentrated extract was transferred quantitatively to a 10 ml volumetric flask with light petroleum ether. If desired, the lipids may be saponified, and the unsaponifiable matter may be removed prior to isomerization. This extract can be stored in a refrigerator for several months without marked changes of PUFA.

d. Determination of the amount of total lipids and naturally conjugated PUFA

For measuring the total lipids in each sample, a part of the extract (usually 5.00 ml) was transferred to a glass stoppered vessel and evaporated in an atmosphere of inert gas, and the residue was estimated.

To determine naturally conjugated PUFA, the residue after estimation of total lipids was redissolved in light petroleum ether and made the volume to 10 ml. Absorption of the solution was estimated at 233, 262, 268, 274, 308, 315 and 322 $m\mu$ against a blank solvent.

e. Isomerization

Exact 0.50 ml of the extract in light petroleum ether was transferred to the vessel for isomerization. The solvent was evaporated under a flow of nitrogen, 1 ml of 95% ethanol was added, 1.1 g (0.85 ml) of ethylene glycol-potassium hydroxide reagent was measured into the vessel using a syringe, then mixed thoroughly. Under nitrogen flow of about 2 ml per second, the tube was placed in the hot oil bath at $180^{\circ} \pm 0.5^{\circ}\text{C}$ and left there for exactly 20 minutes, then cooled in cold water. It was diluted to 5.0 ml by methanol. At the same time a blank sample with 0.50 ml of light petroleum ether instead of the petroleum ether extract was treated by the same procedure. Against this blank sample the absorptions of the isomerized sample at 213, 223, 233, 262, 268, 274, 308, 315, 322, 338, 346, 354, 369, 375 and 381 $m\mu$ were investigated. At this investigation the slit width should be less than 1.0 mm for 233 $m\mu$, less than 0.5 mm for 268 $m\mu$ and less than 0.2 mm for 375 $m\mu$. If the absorption exceeds 1.5, the sample and the blank solution must be diluted and the absorption estimated again from 213 $m\mu$.

f. Calculation

For the calculation of naturally conjugated PUFA, the extinction coefficient k'_{λ} should be obtained by the formula from the absorptions of redissolved lipids above

measured D'' .

$$k_1 = D_1''/CL \quad C: \text{the concentration of solution in g/l}$$

L: the light path of the cell cm

Then the corrected extinction coefficients k_n should be calculated by the following formulae²¹⁾. The subnumber n indicates the number of double bonds.

$$k_2 = k'_{233} - k_0 \quad k_0 = 0.07 \text{ in the case of esters}$$

 $k_0 = 0.03$ in the case of free acids.

$$k_3 = 2.8 \{k'_{268} - (k'_{262} + k'_{274})/2\}$$

$$k_4 = 2.5 \{k'_{315} - (k'_{308} + k'_{322})/2\}$$

From this k_n the quantities of naturally conjugated PUFA C_n could be calculated by the following formulae²¹⁾.

$$C_2 = \% \text{ of conjugated dienoic acid} = 0.84 \text{ k}_2$$

$$C_3 = \% \text{ of conjugated trienoic acid} = 0.47 \text{ } k_3$$

$$C_4 = \% \text{ of conjugated tetraenoic acid} = 0.45 \text{ } k_4$$

In the case of serum or plasma the absorption measured D'_λ should be multiplied by the dilution rate (for example, if 2.00 ml of serum was extracted and the volume was made to 10.0 ml, then the solution was diluted two times for measurement, the dilution rate was $5 \times 2 = 10$), and D'_λ was gained. This D'_λ was used instead of k'_λ of the above formulae, and the naturally conjugated acids were calculated by the unit of mg/dl.

By this calculation C_n can be obtained but the quantities of naturally conjugated PUFA are so small as compared with the unconjugated that they can be neglected in practice.

To calculate the unconjugated natural PUFA, the following corrections of two steps should be made to avoid the errors discussed in the next section. The extinction coefficient k'_λ was introduced from the absorption D'_λ determined after isomerization by the formula $k=D/CL$.

Then the primarily corrected extinction coefficients k_n were calculated by the following formulae.

$$k_6 = 1.5 \{k'_{375} - 1/2(k'_{369} + k'_{381})\}$$

$$k_5 = 2 \{k'_{346} - 1/2(k'_{338} + k'_{354})\}$$

$$k_4 = 2 \{k'_{315} - 1/2 (k'_{308} + k'_{322})\}^{24)}$$

$$k_3 = 4 \{k'_{268} - 1/2(k'_{262} + k'_{274})\}^{24)}$$

From this k_n the secondarily corrected extinction coefficients K_n should be calculated using the following formulae.

$$K_6 = k_6$$

$$K_5 = k_5 + 0.62k_6$$

$$K_4 = k_4 + 0.316k_5 + 0.552k_6$$

$$K_2 = k_3 + 0.313k_4 + 0.291k_5 + 1.024k_6$$

K_n could be regarded as the true absorptions of isomerized PUFA themselves, the differences between K' and K_n were regarded as background absorptions. Suitable formulae for K_2 were not obtained. K_2 was calculated as follows. As $k_{a\lambda}$ were the background absorptions, the following formulae were obtained.

$$k'_{268} - K_3 = k_{a268}$$

$$k'_{315} - K_4 = k_{a315}$$

$$k'_{346} - K_5 = k_{a346}$$

$$k'_{375} - K_6 = k_{a375}$$

A curve was drawn on graph paper through these points of $k_{a\lambda}$ and made it an asymptote of the other curve drawn through k_{213} , k_{223} etc.. This curve showed the background absorption and on the curve the background absorption at 233 $m\mu$ (k_{a233}) was measured, then K_2 could be calculated from the formula,

$$K_2 = k'_{233} - k_{a233}.$$

From these K_n values the quantities of PUFA were calculated by the following formulae.

$$\% \text{ Di (calculated as linoleic acid)} = 1.087K_2 - 0.615K_3 - 0.131K_4 - 0.122K_5 - 0.203K_6$$

$$\% \text{ Tr (calculated as linolenic acid)} = 1.266K_3 - 0.813K_4 + 0.242K_5 - 1.70K_6$$

$$\% \text{ Tt (calculated as arachidonic acid)} = 1.456K_4 - 1.362K_5 - 0.391K_6$$

$$\% \text{ Pt (calculated as eicosapentaenoic acid)} = 1.49K_5 - 1.556K_6$$

$$\% \text{ Hx (calculated as docosahexaenoic acid)} = 4.00K_6$$

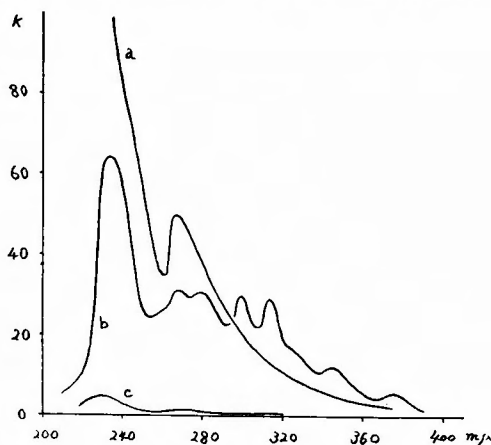
When in determining the PUFA in serum or plasma, the values of D'_λ multiplied by the dilution rate were used instead of k'_λ , then the quantities were in units of mg/dl.

g. Discussion

When the original method of HOLMAN and HAYES was examined in our laboratory, it was found to have some imperfections.

As background absorption the original method used the absorption of the extract of light petroleum ether, and consequently sometimes the background absorption was too high and the calculation was not possible (Fig. 1). For this point, the author's

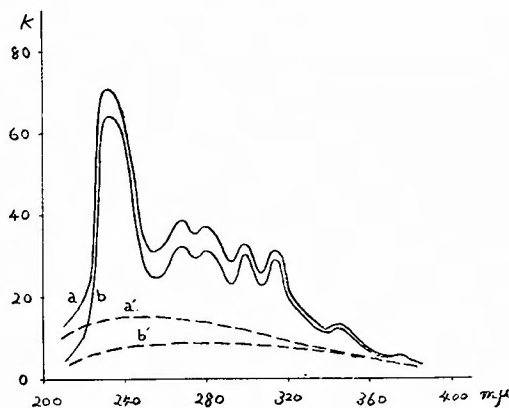
Fig. 1 PUFA absorptions in 3 different processes



- a. Absorptions of petroleum ether extract
- b. Absorptions after alkaline isomerization
- c. Absorptions of naturally conjugated PUFA after redissolving

investigation discovered that the abnormal absorption of the petroleum ether solution was caused by the concentration process at the time of distillation at reduced pressure. To avoid this error the solvent was evaporated completely once, the oxidation of PUFA being prevented by a flow of nitrogen, and then redissolved. By this method the natural conjugated PUFA could also be determined (Fig. 1). But this modification for background absorption was not enough to eliminate all the errors of the method. Because duplicate measurements of the same extract had some errors, and these errors were manifested especially at shorter lengths of the spectrum (Fig. 2).

Fig. 2



Duplicate measurement of PUFA absorptions

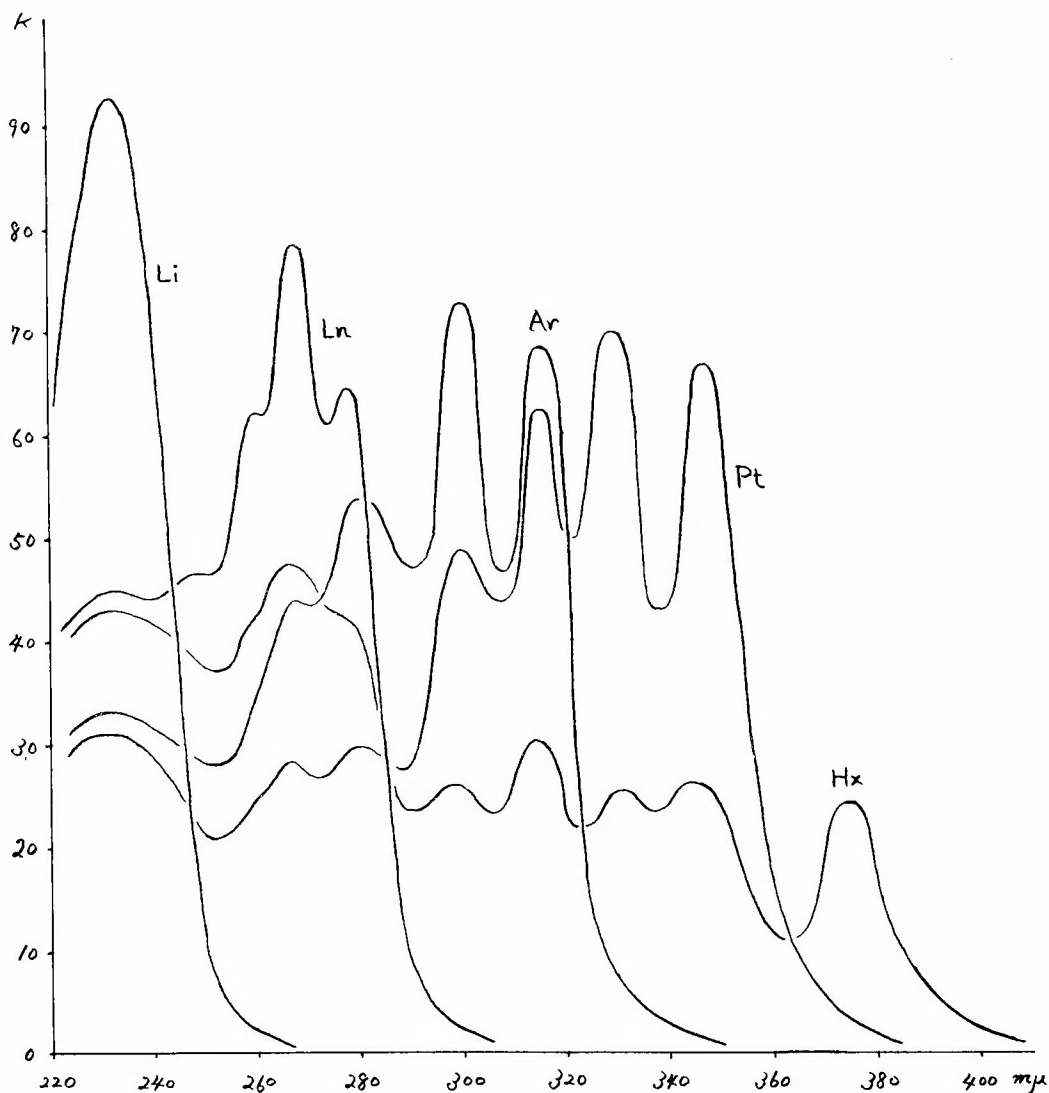
a' and b' : Background absorptions

This error was probably caused by changes in the reagent itself under the conditions of the reaction. Unless this error is corrected, the reproducibility of this method is not satisfactorily high, and the error is up to 10% as reported by HOLMAN and HAYES³⁰⁾.

To correct the error, because the background absorption which caused the error had no local maximal or minimal spectrum, as is seen in Fig. 2, it was believed that the corrections which have so far used in the determination of various plant oils could be used. But the correction was not of the method of HOLMAN and HAYES but only of k_3 and k_4 . So a correction was still needed for higher k_n . For establishing the constant of the correction formulae, absorption spectra of standard PUFA after being processed by this method were needed. But it was difficult to obtain pure standards of PUFA, so the standard absorption spectra were drawn based on the table of k of HOLMAN and HAYES (Table 1)²¹⁾³⁰⁾ and referring to the spectra of HERB and RIEMENSCHNEIDER¹⁾, and HAMMOND and LUNDBERG³⁶⁾ (Fig. 3). With these standard spectra and the tables of BRICE, SWAIN, HERB, NICHOLS and RIEMENSCHNEIDER²⁴⁾, the background factors were established, as described above, for k_3 "4", for k_4 "2", for k_5 "2" and for k_6 "1.5". The primary correction was done in this way, but it could be applied to only such oils as plant oils which contain fewer acids as the unsaturation increases.

But since in animal fats the absorption of Tt, Pt and Hx are not negligible

Fig. 3 Standard spectra of PUFA (Method of HOLMAN and HAYES)



in the estimation of Di and Tr, the errors are not corrected by this method, because the primary correction was only to the errors of the representative spectral wave lengths of the acids. This can be understood from Table 1, which was the original table of HOLMAN and HAYES, and from Table 2 which shows the k of the acids after the primary correction from the standard absorption spectra (Fig. 3).

To obtain the exact background absorption curve, it is necessary to correct these errors, too. For this purpose a secondary correction was needed. The derivations of the secondary corrections are as follows:

$$K_6 = k_6$$

$$K_5 = k_5 + \{(26.1 - 13.1) / 25.0\} k_6$$

Table 1. Spectral constants for polyunsaturated acids (HOLMAN)
k for free acid

Acid	233	268	315	346	375m μ
Li	92.0				
Ln	44.7	79.0			
Ar	33.2	44.1	68.7		
Pt	30.8	27.5	62.8	67.1	2.85
Hx	43.3	48.6	31.2	26.1	25.0

Table 2. Constants corrected by the primary correction
k for free acid

Acid	233	268	315	346	375m μ
Li	92.0				
Ln	44.7	79.0			
Ar	33.2	22.6	68.7		
Pt	30.8	8.0	41.6	67.1	
Hx	43.3	23.0	17.4	13.1	25.0

$$K_4 = k_4 + \{(62.8 - 41.6) / 67.1\} k_5 + \{(31.2 - 17.4) / 25.0\} k_6$$

$$K_3 = k_3 + \{(44.1 - 22.6) / 68.7\} k_4 + \{(27.5 - 8.0) / 67.1\} k_5 + \{(48.6 - 23.0) / 25.0\} k_6$$

With these corrections, the reproducibility from the same extract was within 1% in respect to Di, Tr and Tt, and the errors in the extracting process were less than 5%. As for Pt and Hx, the errors, except for those of the extraction process were less than 3%. In measuring Pt and Hx it is desirable to use more samples or more extract for isomerization.

III. THE DETERMINED RESULTS OF PUFA IN VARIOUS SAMPLES

This modification of the method of HOLMAN and HAYES was used in several clinical and experimental studies.

A) METHOD OF EXPERIMENTS

1. Clinical Experiments

a. Serum

The PUFA were determined in the fasting serum of patients admitted to the Second Surgical Division of the Kyoto University Hospital. The patients were 23 cases of gastric cancer, 6 of esophageal cancer, 3 of bronchial cancer and 2 of pancreatic cancer, and as a control 9 cases of gastric ulcer, 4 of duodenal ulcer and 5 healthy men were chosen.

b. Liver

Of these patients 13 with gastric cancer, 6 with gastric ulcer, and 4 with duodenal ulcer were operated on. At operation, liver biopsies were done and the PUFA of the liver were determined.

c. Gastric cancer and gastric mucosa

Eight stomachs were removed for gastric cancer, the lipids were extracted from

the tumour and the healthy mucosa and the PUFA were determined. Healthy mucosa of stomachs removed at gastrectomy for gastric ulcer was also examined.

2. Animal Experiments

Four groups of Wistar-strain male albino rats were fed various diets from their first to 7th month, and five rats from each group were examined.

They were anesthetized by nembutal in a postabsorptive state and sacrificed by bleeding from the aorta. Samples were taken from the lung, heart, kidney, liver, adrenal gland and serum, and the PUFA were determined.

a. Group I

These rats were fed refined rice grain with 20 ml/kg of refined sesame oil plus a little fish, cabbage and vitamins. This group received more than 200 mg of PUFA per day.

b. Group II

For the first 3 months this group was fed the same diet as group I except that the sesame oil contained 3% butter-yellow (Para-dimethyl-aminoazobenzol)³⁷⁾. After three months the liver showed precancerous changes. During the last 3 months they were fed by the same diet as group I, but a typical hepatoma developed in the fifth month mostly in the ventral lobe.

c. group III

This group was fed the same diet as group I except that refined sesame oil was replaced by the same volume of olive oil, which has less PUFA. During the last 3 months the olive oil was omitted, i. e. a fat-deficient diet was fed to the animals. The PUFA intake was less than 5 mg per day.

d. Group IV

This group was fed the same diet as group III except that to the olive oil was added 3% butter-yellow for the first 3 months. For the last 3 months the olive oil and butter-yellow were omitted and the diet was the same as that of group III. This group also developed hepatoma in the fifth month.

B) RESULTS

1. Results of Clinical Experiments

a. Quantities of PUFA in serum

As shown in Table 3 the Di in the serum of healthy men averaged 60.0 mg/dl, but in gastric and duodenal ulcer cases it was decreased, and in patients with gastric, bronchial, pancreatic and esophageal cancer it was even more decreased. A similar decrease in Tr, Tt and Hx occurred, but the Pt level did not vary with the kinds of diseases.

b. Quantities of PUFA in liver

As shown in Table 3, Di, Tr, Tt and Hx were highest in cases of gastric ulcer, and were decreased in cases of duodenal ulcer. In cases of gastric, pancreatic and esophageal cancer they were more decreased.

c. Quantities of PUFA in gastric mucosa and gastric cancer tissue

As seen in Table 3 the Di, Tr, Tt and Hx of healthy portions of gastric mucosa in cases of gastric cancer were clearly decreased as compared with those in gastric

Table 3. Levels of PUFA in various diseases

Sample	Diseases	Cases	Di	Tr	Tt	Pt	Hx	
Serum	Healthy	5	60.0	9.5	17.6	3.5	9.4	} mg/dl
	Ulcus ventr.	9	51.0	6.3	13.7	3.8	6.3	
	Ulcus duod.	4	47.8	3.3	9.8	3.4	7.3	
	Carc. ventr.	23	44.9	4.1	9.3	2.5	4.5	
	Carc. bronch.	3	42.1	4.3	9.6	1.9	1.0	
	Carc. panc.	2	41.7	6.2	8.7	5.8	13.2	
	Carc. oesoph.	6	41.0	1.7	9.1	3.8	5.4	
Liver	Ulcus ventr.	6	0.50	0.09	0.36	0.03	0.42	} %
	Ulcus duod.	4	0.50	0.05	0.23	0.03	0.31	
	Carc. ventr.	13	0.38	0.06	0.22	0.08	0.21	
	Carc. panc.	1	0.40	0.03	0.11	0.02	0.16	
	Carc. oesoph.	1	0.37	0.05	0.13	0.02	0.27	
Gastric mucosa	Ulcus ventr.	5	0.30	0.12	0.38	0.03	0.16	} %
	Carc. ventr.	8	0.18	0.07	0.27	0.03	0.15	
Tumour	Carc. ventr.	8	0.17	0.06	0.20	0.02	0.10	%

Table 4. Effects of tumour and diet on tissue PUFA in rats

Sample	Group	No.	Di	Tr	Tt	Pt	Hx	
Serum	I	5	25.5	0.02	35.4	0.16	0.88	} mg/dl
	II	5	17.4	0.48	19.6	0.52	1.52	
	III	5	23.1	0.01	29.7	0.58	3.72	
	IV	5	12.9	1.82	23.5	3.11	2.22	
Liver	I	5	0.49	0.01	0.70	0.00	0.22	} %
	II	5	0.30	0.06	0.33	0.01	0.11	
	III	5	0.35	0.03	0.35	0.00	0.19	
	IV	5	0.30	0.05	0.32	0.00	0.20	
Hepatoma	III	5	0.17	0.01	0.16	0.01	0.01	} %
	IV	5	0.17	0.04	0.21	0.01	0.05	
Adrenal	I	5	1.18	0.07	1.81	0.14	0.14	} %
	II	5	0.61	0.12	0.84	0.13	0.11	
	III	5	0.55	0.01	1.35	0.06	0.11	
	IV	5	0.55	0.19	1.17	0.12	0.13	
Heart	I	5	0.45	0.05	0.34	0.28	0.08	} %
	II	5	0.31	0.11	0.25	0.00	0.06	
	III	5	0.22	0.01	0.20	0.00	0.09	
	IV	5	0.27	0.02	0.28	0.00	0.15	
Lung	I	5	0.29	0.00	0.24	0.03	0.20	} %
	II	5	0.01	0.03	0.09	0.03	0.03	
	III	5	0.11	0.00	0.17	0.01	0.04	
	IV	5	0.07	0.01	0.17	0.02	0.02	
Kidney	I	5	0.40	0.01	0.52	0.01	0.03	} %
	II	5	0.28	0.01	0.47	0.00	0.03	
	III	5	0.32	0.02	0.45	0.01	0.05	
	IV	5	0.38	0.00	0.41	0.03	0.06	

Group I High PUFA diet

Group II Low PUFA diet

Group III High PUFA diet with butter-yellow

Group IV Low PUFA diet with butter-yellow

ulcer. The PUFA were determined in gastric cancer tissue, and Di, Tr, Tt and Hx were markedly decreased as compared with the healthy gastric mucosa.

2. Results of Animal Experiments

a. Quantities of PUFA in serum

As seen in Table 4, Di in serum was highest in group I, next in group III,

then in group II and lowest in group IV. Tt values showed similar changes, but Pt and Hx did not. A marked increase in Tr was seen in group II and IV, due probably not to increased linolenic acid but to increased 5, 8, 11-eicosatrienoic acid which could not be differentiated from linolenic acid by this method; this increase occurred especially when there was a marked PUFA deficiency.

b. Quantities of PUFA in healthy areas of liver

As seen in Table 4, the PUFA of healthy portions of liver were equal to those in the serum. That is, Di and Tt were maximum in group I, next in group III, then group II and lowest in group IV. Tr was markedly increased in groups II and IV, indicating extreme PUFA deficiency. Pt and Hx were variable.

c. Quantities of PUFA in hepatoma tissue

These values were determined in groups II and IV which developed hepatoma experimentally. As seen in Table 4, there was no significant difference between the two groups. But compared with healthy livers, Di, Tr and Tt decreased to almost half, and Hx also decreased markedly.

d. Quantities of PUFA in the adrenal gland

As shown in Table 4, the adrenal gland contained more PUFA than the other organs, especially Di and Tt. Di and Tt were highest in group I, next in group III and lowest in groups II and IV. Tr was markedly increased in groups II and IV, but Pt and Hx were variable.

e. Quantities of PUFA in the heart

As seen in Table 4, the quantities of PUFA in heart muscle decreased as in serum, but the differences between the groups were smaller than in the other organs.

f. Quantities of PUFA in lung

As seen in Table 4, Di and Tt were decreased in the order group I, III, IV and II. The PUFA of the lung thus seem to be more influenced by the quantity of PUFA ingested than by the tumour. Tr was increased in groups II and IV, as in the other organs.

g. Quantities of PUFA in kidney

The quantities of PUFA in kidney showed, as in heart muscle, small differences between the groups. In group I Di and Tt were maximum.

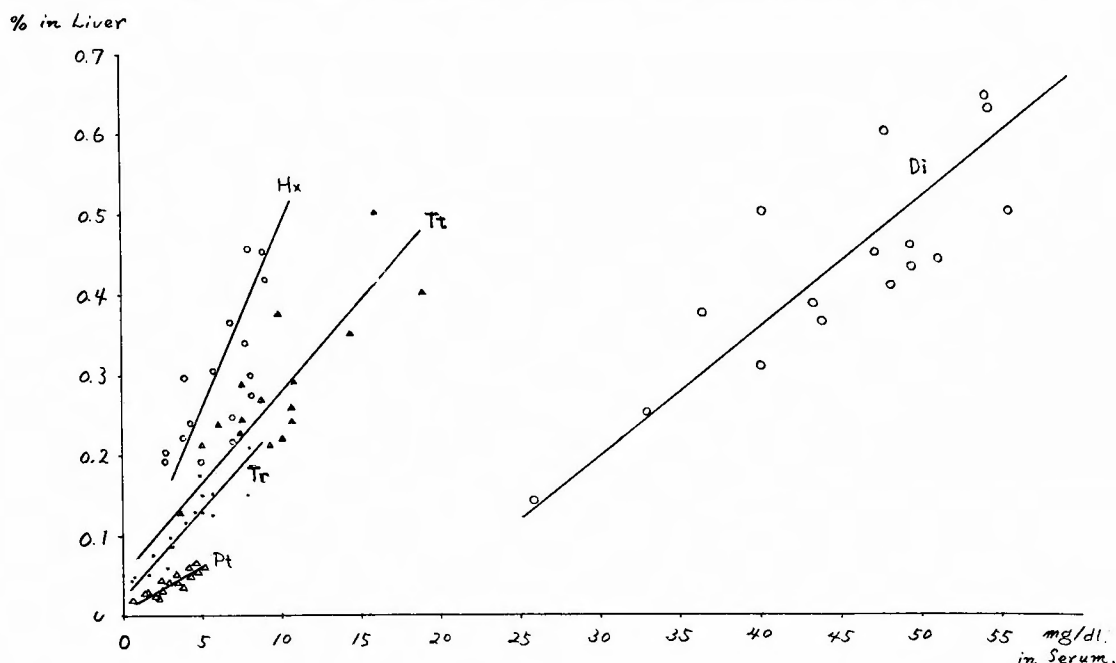
IV. DISCUSSION AND SUMMARY

As was clearly shown in the clinical experiment the quantities of PUFA in serum was highest in healthy men except for Pt. Patients with gastric and duodenal ulcer on limited diets because of the disease had reduced amount of serum PUFA, and in various cancers they were even more decreased: Di and Tr to 70% and Tt to 50% of normal. These changes in serum PUFA paralleled the liver PUFA in the fasting condition, and the Di, Tr, Tt and Hx in the livers of patients with cancer were reduced to about 50% of that in ulcer patients.

The relation between serum and liver PUFA is shown in Fig. 4: all five acids have positive correlations, though each acid shows several differences. This table suggests that Di and Tr are consumed in the liver and Tt and Hx are synthesized

in liver. So in the liver Tt is synthesized from Di⁽¹⁴⁾⁽¹⁵⁾, and Hx from Tr as has already been reported^{(10)~(12)}. But the synthesis of Pt from Tr described by HOLMAN and WIDMER probably takes place mostly in extrahepatic tissues. The fact that each acid of serum and liver PUFA is in almost linear correlation (Fig. 4), indica-

Fig. 4 Relation between liver and serum PUFA concentrations in men



tes that the author's modification of the method of HOLMAN and HAYES is an excellent one which can apply clinically.

It has been clearly demonstrated that PUFA in patients with cancerous diseases are decreased, but whether this is due to increased consumption or decreased supply was not clear from this data, so animal experiments were done on rats with experimentally produced hepatomas. Di and Tt was decreased in various organs, and hepatoma tissue showed much lower values of Di and Tt than healthy liver. This facts indicate that probably the presence of tumour causes increased consumption of PUFA. Fat-deficient diets also caused a similar PUFA pattern.

The PUFA in various organs of rats were compared with those of men; liver PUFA were similar, but serum PUFA of rats less than those of men except for Tt, which was more. PUFA were especially high in the adrenals, as already described by NAGASE of our Division. The PUFA pattern in heart muscle and kidney was similar, and varied less with disease or diet, than AAES-JØRGENSEN and HOLMAN have reported⁽³⁹⁾. Lung PUFA were more changeable than the other organs indicating that lungs play a role in fat metabolism.

As described above, in individuals with tumours the PUFA are decreased. This confirms the studies of HAVEN⁽³²⁾, and of SUEYOSHI and MIURA⁽³³⁾. To clarify whether PUFA deficiency inhibits the growth of tumours or not, more detailed experiments

were carried out on the PUFA in various organs of rats with experimentally induced hepatoma fed fat-deficient diets. In these rats the growth of hepatoma caused by butter-yellow was not inhibited, but was almost the same as in rats fed PUFA sufficient diets.

This fact indicates that in a fat-deficient state, PUFA which are necessary for the growth of tumours were supplied from PUFA of various organs. In the organs of these rats Di, Tt and Hx were more decreased than in rats with tumours fed PUFA sufficient diets. When PUFA were extremely deficient, the typical pattern of decreased Di, Tt and Hx and increased Tr developed. And this pattern was most typical and apparent in the PUFA of the liver and of the adrenal gland, which contains much PUFA in the normal state. This increase of Tr is probably caused by the increase not of linolenic acid but of 5, 8, 11-eicosatrienoic acid which increases in states of PUFA deficiency³⁸⁾

V. CONCLUSION

1) To determine PUFA in tissues under various conditions the method of HOLMAN and HAYES was examined and a modified micromethod was established. This method has an excellent reproducibility of $\pm 1\%$, and of $\pm 5\%$ including the errors of extraction. The modified method added two steps of correction to the original method.

2) When compared with the serum PUFA of healthy men, the serum PUFA of patients with gastric, and duodenal ulcer were clearly decreased, and with various cancers were even more decreased. This same decrease was seen in liver PUFA.

3) To examine whether this is due to increased consumption of PUFA in tumour tissue experimental investigations were done. And it was proved that the existence of tumour caused the decrease in PUFA in various organs by the increased PUFA consumption in rapid tumour growth.

4) In the PUFA deficient state, a special pattern of decreased Di, Tt and Hx and of increased Tr occurs. This increase in Tr is probably due to an increase not of linolenic acid but of 5, 8, 11-eicosatrienoic acid as reported by MEAD et al.

5) The PUFA-deficient pattern was typically and clearly recognized in the PUFA of the liver and of the adrenal gland, which contains much more PUFA in the normal state than do the other organs.

6) In various organs the greatest value of PUFA per unit volume was seen in the adrenal gland, suggesting a closed relation between the quantity of PUFA in the gland and adrenocortical capacity.

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和文抄録

不飽和脂酸の微量定量法の吟味

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1) 各種条件下の生体内不飽和脂酸殊に不可欠脂酸の測定に当り、Holman 及び Hayes の原法を再検討し、新たにその微量分光分析法を確立した。本法によれば再現率を±1%程度におさえ、またその抽出操作を含めた再現率を±5%以内に止めることが可能となつた。なお本法は原法に二段階の補正を加えるものである。

2) 血清中の不可欠脂酸含有量は健常者に比べ、胃・十二指腸潰瘍患者では既に明らかに低下しており、更に各種癌疾患患者に於ては一層低下している。また、肝臓中の不可欠脂酸含有量についても全く同様の傾向を認めた。

3) この原因が、生体内に存在する発育の旺盛な腫瘍組織の不可欠脂酸消費量の亢進によるものであるかどうかを更に実験に匡し、確かに腫瘍の存在はその著しい消費を招き、ために生体内各臓器中の不可欠脂酸含有量を低下させる事実を知つた。

4) 生体内不可欠脂酸の欠乏に際しては、Di, Tt 及び Hx の減少と同時に Tr の増加を来するという特有の Pattern を示すが、この Tr の増加はリノレン酸の増加にもとづくものではなく、恐らく Mead のいうように、生体内不可欠脂酸の欠乏に際して著増する 5,8,11-Eicosatrienoic acid の増加によるものであろう。

5) 以上のような生体内不可欠脂酸の欠乏に際して認められる特有な Pattern は平素から不可欠脂酸を多量に含有する副腎、肝臓に於て最も顕著且つ典型的に認められる。

6) 生体内各臓器中で単位容積当りの不可欠脂酸保有量の最も大きな臓器は副腎であり、その不可欠脂酸保有量と副腎皮質機能予備能力との間には密接な相関性のあることが臆測される。

7) 以上の臨床的並びに基礎的研究成績を通じて、私の改良した不飽和脂酸定量法が極めて優秀な測定法であることを立証し得た。